

## LETTERS

# Antiviral immunity in *Drosophila* requires systemic RNA interference spread

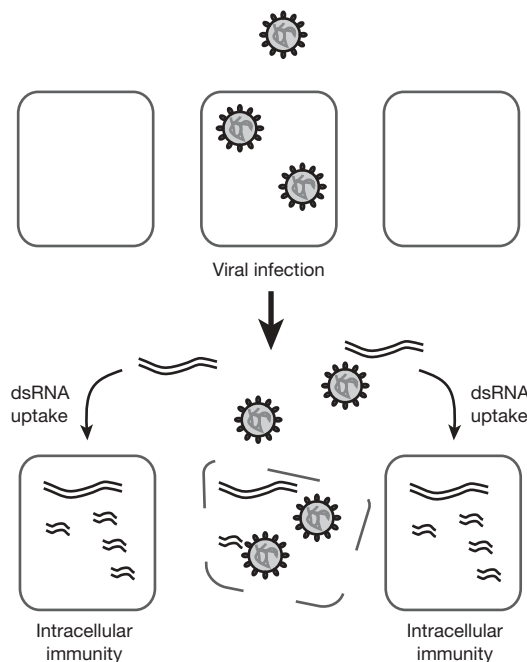
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Multicellular organisms evolved sophisticated defence systems to confer protection against pathogens. An important characteristic of these immune systems is their ability to act both locally at the site of infection and at distal uninfected locations<sup>1–4</sup>. In insects, such as *Drosophila melanogaster*, RNA interference (RNAi) mediates antiviral immunity<sup>5–7</sup>. However, the antiviral RNAi defence in flies seems to be a local, cell-autonomous process, as flies are thought to be unable to generate a systemic RNAi response<sup>8</sup>. Here we show that a recently defined double-stranded RNA (dsRNA) uptake pathway<sup>9</sup> is essential for effective antiviral RNAi immunity in adult flies. Mutant flies defective in this dsRNA uptake pathway were hypersensitive to infection with *Drosophila* C virus and Sindbis virus. Mortality in dsRNA-uptake-defective flies was accompanied by 100- to 10<sup>5</sup>-fold increases in viral titres and higher levels of viral RNA. Furthermore, inoculating naked dsRNA into flies elicited a sequence-specific antiviral immune response that required an intact dsRNA uptake pathway. These findings suggest that spread of dsRNA to uninfected sites is essential for effective antiviral immunity. Notably, infection with green fluorescent protein (GFP)-tagged Sindbis virus suppressed expression of host-encoded GFP at a distal site. Thus, similar to protein-based immunity in vertebrates, the antiviral RNAi response in flies also relies on the systemic spread of a virus-specific immunity signal.

On the basis of the recent identification of a dsRNA uptake pathway in flies<sup>9,10</sup>, we hypothesized that dsRNA produced and released from infected cells can be taken up locally, and perhaps at distal sites, to establish systemic pre-existing immunity in uninfected cells (Fig. 1). We thus examined whether naked dsRNA can mediate systemic RNAi spread by inoculating flies with dsRNA corresponding to two different regions of the Sindbis virus genome (dsSin1 and dsSin2, Supplementary Fig. 1a; see also Fig. 2a). Two days after dsRNA inoculation, flies were infected with a recombinant Sindbis virus expressing GFP (Sindbis-GFP virus, Supplementary Fig. 1a). Notably, inoculation with dsSin1 and dsSin2 markedly reduced accumulation of GFP as determined by fluorescence microscopy and immunoblotting (Fig. 2b, c, lanes 7–11 and 18–22); control buffer had no effect on virus replication (Fig. 2b, c, lanes 2–6 and Supplementary Fig. 1b). This inhibitory response was sequence specific because flies inoculated with dsRNA corresponding to *Drosophila* C virus (DCV) genome showed no effect on Sindbis virus replication (Fig. 2b, c, lanes 13–17). Furthermore, inoculation of dsRNA corresponding to DCV (dsDCV) efficiently protected wild-type flies against DCV infection, but not against Sindbis virus (Supplementary Fig. 2a). The antiviral effect of exogenous dsRNA inoculation required a functional RNAi machinery as Dicer 2 and Argonaute 2 null mutant flies

(*Dcr2*<sup>−/−</sup> and *Ago2*<sup>−/−</sup>) were unable to mount an effective antiviral response (Fig. 2d and Supplementary Fig. 2a). In addition, wild-type flies accumulated short interfering RNAs (siRNAs) derived from injected dsRNA (Supplementary Fig. 2c). We conclude that inoculation of dsRNA initiates a bona fide, specific RNAi response that protects flies against virus infection.

Serial dilutions of dsSin2 indicated that very low concentrations of injected dsRNA sufficed to mount a very strong response (Fig. 2e). Accordingly, we observed reduced viral replication even after inoculation of 5 pg of dsRNA (equivalent to 1.5 × 10<sup>5</sup> molecules of dsSin2, Fig. 2e, lanes 17–20). Of note, whereas the maximal dose of dsSin2 (5 ng) elicited an inhibitory response that lasted 5 days (Fig. 2e, lanes 5–8), inoculation of a lower dose produced a shorter period of immunity (Fig. 2e, compare lanes 5–8 with 9–12, 13–16 and 17–20).

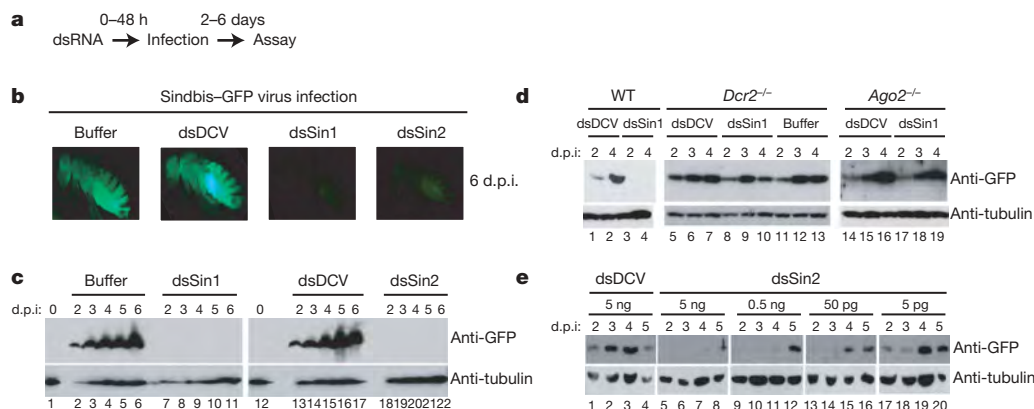


**Figure 1 | Model for systemic RNAi viral immunity in *Drosophila melanogaster*.** Upon viral infection, virus-specific dsRNAs (for example, replication intermediates) are generated during the initial rounds of virus replication. After cell death or lysis, dsRNAs are taken up and processed by uninfected cells to protect them from subsequent infection, thereby preventing virus spread.

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**Figure 2** | *In vivo* dsRNA immunization provides sequence-specific antiviral protection in *D. melanogaster*. **a**, Immunization protocol. **b**, Wild-type flies infected with Sindbis-GFP virus two days after intrathoracic injection of dsRNA against *Drosophila* C virus (DCV, 442 bp long, corresponding to the viral polymerase between nucleotides 5589–6030), dsRNA against Sindbis virus non-structural proteins 1 and 2 (dsSin1, 901 bp long, corresponding to nucleotides 1211–2112) or dsRNA against Sindbis virus corresponding to the non-structural proteins 3 and 4 (dsSin2, 954 bp long, corresponding to nucleotides 5485–6439). Buffer,

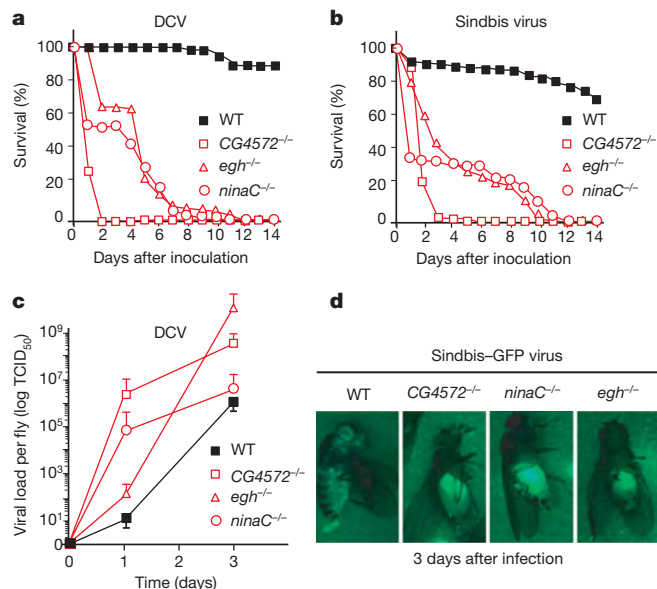
control injection; d.p.i., days post infection. Sindbis-GFP virus replication was monitored by GFP production. **b**, Fluorescence images. **c**, Western blot with an anti-GFP antibody. **d**, Sindbis-GFP virus challenge in wild-type, homozygous *Dcr2*<sup>L811fsX</sup> (*Dcr2*<sup>-/-</sup>) and homozygous *Ago2*<sup>414</sup> (*Ago2*<sup>-/-</sup>) flies. **e**, dsRNA immunization protects in a dose-dependent manner. Flies were inoculated with dsRNA (dsSin2) directed against Sindbis-GFP. Virus replication over time (2–5 d.p.i.) was monitored by western blotting using an anti-GFP antibody.

This observation underscores the efficiency and persistence of the dsRNA-mediated antiviral immunity in *Drosophila*, and supports the idea that exogenous dsRNA can initiate an RNA silencing response in flies, albeit without the RNAi amplification mechanism observed in plants and nematodes<sup>11,12</sup>.

We previously described that dsRNA is taken up in *Drosophila* S2 cells by an active pathway, involving receptor-mediated endocytosis<sup>9</sup>. To examine whether this pathway is involved in the antiviral RNAi response mechanism we selected three genes implicated by the previous analysis in dsRNA uptake: *egghead* (*egh*), encoding a seven-transmembrane-domain glycosyltransferase; *ninaC*, coding for a protein involved in vesicle transport; and a gene of unknown function, *CG4572* (Supplementary Fig. 2b). Although viability and fertility of homozygous *egh*<sup>EP804</sup>, *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> mutant flies did not differ significantly from wild type, all three mutants were hypersensitive to DCV or Sindbis virus infection. In these dsRNA uptake-defective flies, we observed an earlier onset of disease (Fig. 3a, b). After infection, median survival of homozygous *egh*<sup>EP804</sup>, *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> flies was approximately 5–8 days, compared with more than 14 days in wild-type flies, and the 50% lethal dose (LD<sub>50</sub>) in *CG4572*<sup>c05963</sup> flies was ninefold lower than in wild-type flies (not shown).

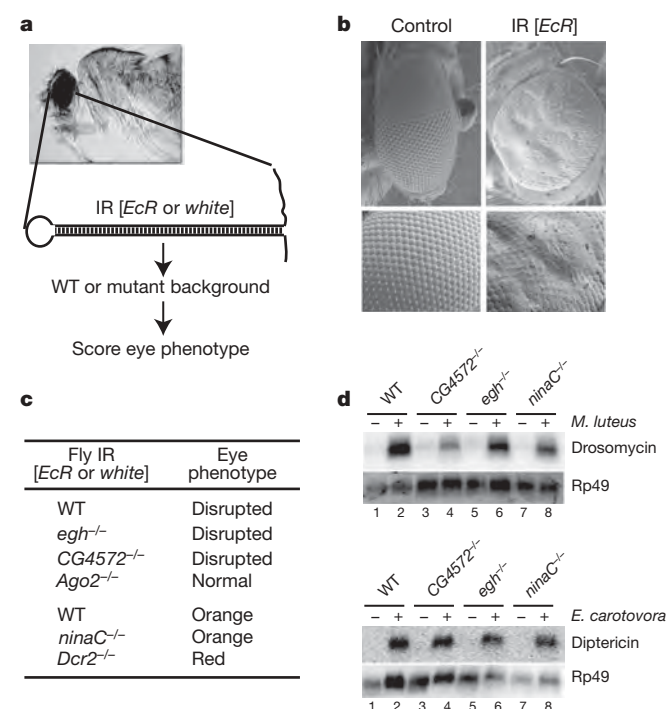
An important consideration when studying viral sensitivity in animals defective for components of a major cellular pathway, such as endocytosis or intracellular transport, is that enhanced death after viral infection may be caused by a decrease in fitness or general health of the mutant animal, and not by a direct antiviral activity of the deleted component. To establish whether the increased mortality of *egh*, *ninaC* and *CG4572* mutant flies stems from their inability to control virus replication, we determined viral loads (Fig. 3c). Even at early time points after infection, before the onset of disease, DCV titres were 100- to 10<sup>5</sup>-fold higher in homozygous *egh*<sup>EP804</sup>, *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> flies compared to wild-type controls (Fig. 3c). The increase in viral titres in mutant flies was mirrored by a marked increase in viral RNA levels. Whereas viral RNA was barely detected in wild-type flies before day 5, it was clearly observed at 24 h after infection in homozygous *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> mutant flies, and by 48 h it accumulated at much higher levels in these mutants than in wild-type flies (Supplementary Fig. 3a). We examined the role of the dsRNA uptake pathway on virus replication further by monitoring Sindbis-GFP virus tissue tropism. In wild-type flies, GFP fluorescence was barely detected 3 days after infection and accumulated

in discrete puncta throughout the fly. In contrast, in homozygous *egh*<sup>EP804</sup>, *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> mutants, GFP accumulated within a large structure in the abdomen of the animal and at much higher levels than in wild-type flies (Fig. 3d and Supplementary Fig. 3b). These results indicate that the enhanced viral susceptibility of *egh*, *ninaC* and *CG4572* mutant flies is due to their inability to control virus replication.



**Figure 3** | Increased viral susceptibility of dsRNA-uptake-deficient mutants. **a**, **b**, Survival of dsRNA uptake mutant flies after virus infection. Homozygous *egh*<sup>EP804</sup> (*egh*<sup>-/-</sup>), *ninaC*<sup>3</sup> (*ninaC*<sup>-/-</sup>), *CG4572*<sup>c05963</sup> (*CG4572*<sup>-/-</sup>) and wild-type flies were injected with 500 TCID<sub>50</sub> (50% tissue culture infectious dose) DCV (**a**) or 500 plaque-forming units Sindbis-GFP virus (**b**) and monitored daily for survival. **c**, DCV replicates at higher levels in dsRNA uptake mutant flies. Flies were injected with 500 TCID<sub>50</sub> DCV, and virus production was monitored over time. At each time point, three pools of five flies were homogenized, and the viral titre in the homogenate was determined by end-point dilution. The error bars report the average  $\pm$  s.d. for at least three independent experiments. **d**, Sindbis-GFP virus replicates at higher levels in dsRNA uptake mutant flies as shown by increased GFP expression in the fat body at 3 days after infection when compared with wild-type flies.

The hyper-susceptibility to virus infection of flies defective in dsRNA uptake is strikingly similar to that previously seen in *Ago2*-defective flies<sup>6</sup>. Therefore, we examined whether the RNAi core function is impaired in *egh*, *ninaC* and *CG4572* mutant flies. Eye-specific silencing of the *Ecdysone receptor* gene (*EcR*) by an endogenously expressed *EcR* hairpin dsRNA leads to abnormal eye structure resulting from impaired corneal lens formation (Fig. 4a, b). Under these conditions, disruption of the core RNAi machinery in homozygous *Ago2*<sup>414</sup> mutant flies suppressed *EcR* RNAi and restored normal eye structure. In contrast, efficient *EcR* RNAi was observed in homozygous *egh*<sup>EP804</sup> and *CG4572*<sup>c05963</sup> flies. Similar experiments monitored RNAi in homozygous *ninaC*<sup>3</sup> flies using the expression of a hairpin dsRNA targeting the *white* gene that causes a decrease of eye pigmentation and orange eye colour in control flies (Supplementary Fig. 4a, b)<sup>14</sup>. Silencing of *white* was suppressed in homozygous *Dcr2*<sup>L811fsX</sup> mutant flies whereas it was fully maintained in homozygous *ninaC*<sup>3</sup> flies (Fig. 4c and Supplementary Fig. 4a, b). We further confirmed this conclusion by injecting dsRNA against the *fushi tarazu* gene (*ftz*)<sup>15</sup> into syncytial embryos before cellularization. Injection of *ftz* dsRNA in wild-type embryos resulted in the expected segmentation defects, namely loss of denticle belts in the cuticle of pre-hatching larvae (*ftz* phenotype; Supplementary Fig. 4c). Injection of *ftz* dsRNA in homozygous *egh*<sup>EP804</sup>, *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> embryos induced the same defects, indicating that RNA silencing proceeded normally in these mutants. In contrast, homozygous *Ago2*<sup>414</sup> control embryos were unable to silence *ftz* expression and thus hatched with a wild-type cuticle (Supplementary Fig. 4c). These results indicate that mutant flies support efficient RNAi silencing if dsRNA uptake is bypassed through expression of dsRNA hairpins intracellularly or by injecting dsRNA into syncytial embryos.



**Figure 4 | Core RNAi machinery and antibacterial immunity are intact in dsRNA uptake mutants.** **a**, Schematic to test the core RNAi machinery integrity. **b**, RNAi processing of an inverted repeat IR [EcR] induced by the GMR-GAL4 driver prevents the formation of the corneal lens (electron microscopy picture, top panels are  $\times 200$  original magnification, bottom panels are  $\times 500$  original magnification). **c**, Monitoring corneal lens formation and eye colour in transgenic flies deficient in the dsRNA uptake pathway. **d**, Susceptibility of dsRNA uptake mutant flies to infection is specific to the viruses, as the dsRNA uptake mutant flies are able to produce antimicrobial peptides in response to an infection by pathogenic Gram-positive and Gram-negative bacteria.

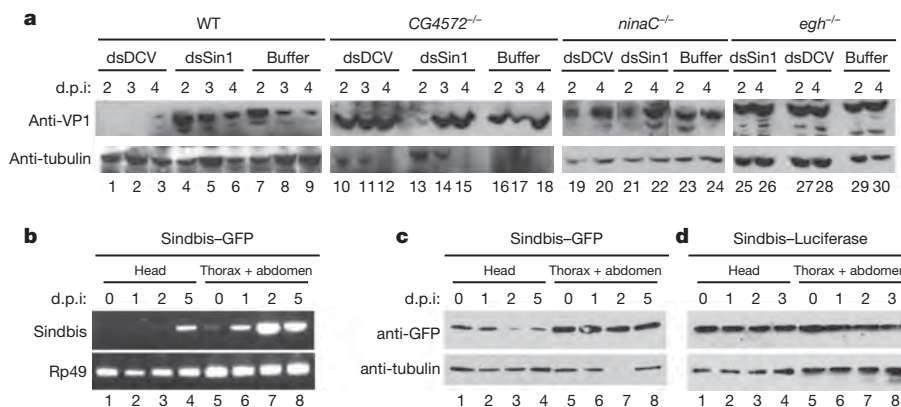
We next examined whether other arms of the immune system were affected in *egh*, *ninaC* and *CG4572* mutants. Insects produce a number of antimicrobial peptides, which are secreted into the haemolymph, in response to immune challenge. These peptides are effective against Gram-negative and Gram-positive bacteria as well as fungi<sup>16</sup>. We determined whether *egh*, *ninaC* and *CG4572* mutant flies can support production of the antimicrobial peptides drosomycin and dipterin in response to septic injury with Gram-positive and Gram-negative bacteria. Drosomycin production was measured after septic injury with *Micrococcus luteus*, a Gram-positive bacterium that signals through the Toll pathway. Production of dipterin was measured after septic injury with *Erwinia carotovora* (also called *Pectobacterium carotovorum*), a Gram-negative bacterium that induces the Imd pathway. Homozygous *egh*<sup>EP804</sup>, *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> flies were able to respond efficiently to bacterial infection (Fig. 4d). Similarly, the JAK/STAT signalling pathway<sup>17</sup> seems to be unimpaired in *egh*<sup>EP804</sup> and *CG4572*<sup>c05963</sup> flies as DCV infection induced normal *vir-1* expression in these mutants (Supplementary Fig. 5). Thus, defects in cellular components that abrogate dsRNA uptake and its ensuing antiviral immunity do not generally impair other arms of the fly innate immune system.

We hypothesize that the dsRNA uptake pathway underlies systemic antiviral immunity, which is required to control virus replication. We thus examined whether dsRNA inoculation in *egh*, *ninaC* and *CG4572* mutant flies was able to elicit the protective immunity observed in wild-type flies (Fig. 2). Indeed, whereas inoculation of DCV dsRNA markedly reduced DCV replication in wild-type flies (Fig. 5a, lanes 1–9), homozygous *egh*<sup>EP804</sup>, *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> mutant flies were unable to mount an antiviral response on DCV dsRNA inoculation (Fig. 5a, lanes 10–30). Similarly, the dsRNA uptake pathway was required for protection against Sindbis virus infection by naked dsRNA inoculation (Supplementary Fig. 6). Furthermore, whereas wild-type flies efficiently processed inoculated dsRNA into siRNAs, *egh*<sup>EP804</sup>, *ninaC*<sup>3</sup> and *CG4572*<sup>c05963</sup> mutant flies accumulated siRNAs at much lower levels (Supplementary Fig. 2b).

Our model states that infected cells release viral dsRNA that is subsequently taken up by uninfected cells through the dsRNA uptake pathway, thereby eliciting an antiviral RNAi response. A direct prediction of this model is that during infection, viral-derived dsRNA spreads to induce systemic silencing. To test this prediction we examined whether infection with a Sindbis virus carrying the GFP gene could silence a ubiquitously expressed endogenous GFP at a distal site. After intrathoracic inoculation, Sindbis-GFP virus RNA was readily detected in the thorax and abdomen of Tub-eGFP transgenic flies starting at 1 day after infection (Fig. 5b, lanes 5–8). In contrast, the viral RNA was not detectable in the head until day 5 after infection (Fig. 5b, lanes 1–4). Notably, endogenous GFP expression in the head was significantly reduced already at day 2, despite the absence of any detectable viral replication in this organ (Fig. 5c, lane 3). In contrast, infection with control Sindbis virus carrying a firefly luciferase gene did not silence GFP expression (Fig. 5d). These results indicate that a virus-specific derived RNAi signal spreads from the thorax to the head early after infection.

It was previously thought that *Drosophila* is unable to spread systemically an RNAi response, based on observations that endogenously expressed RNA hairpins do not spread from cell to cell<sup>8</sup>. However, we demonstrate that, upon virus infection, infected cells spread systemically a silencing signal that elicits protective RNAi-dependent immunity throughout the organism. Although uninfected *Drosophila* cells seem to lack a constitutive mechanism for systemic RNAi spread, unlike plant and nematode cells, they do have an active and highly efficient mechanism for dsRNA uptake, which we here show is essential for antiviral immunity. Accordingly, dsRNA is normally not released from uninfected cells, but virus infection may induce dsRNA release either through lysis of infected cells or through a virally induced shedding mechanism. We propose that these virally derived dsRNAs are taken up into uninfected cells to generate virus-specific intracellular





**Figure 5 | Systemic spread of dsRNA follows virus infection and is essential for effective antiviral immunity.** **a**, *Drosophila* C virus infection in wild-type flies, and in homozygous *egh<sup>EP804</sup>* (*egh<sup>-/-</sup>*), *ninaC<sup>3</sup>* (*ninaC<sup>-/-</sup>*) and *CG4572<sup>c05963</sup>* (*CG4572<sup>-/-</sup>*) mutant flies inoculated with the indicated dsRNA. DCV replication was monitored by western blotting using an antibody directed against DCV capsid protein VP1. **b–d**, dsRNA produced during virus replication can spread and silence endogenous GFP expressed

immunity that prevents virus spread (Fig. 1). In support of this idea, this specific antiviral response in flies requires both the RNAi core machinery and the recently described dsRNA uptake pathway. Furthermore, simple inoculation of even very low amounts of dsRNA, in the absence of virus infection, can by itself promote a potent antiviral immunity, which is similarly dependent on the RNAi core machinery and the dsRNA uptake pathway. Our previous results indicated that whereas dsRNA is readily taken up by *Drosophila* S2 cells, siRNAs are not efficiently taken up<sup>9</sup>. We thus conclude that systemic spread of a specific antiviral RNAi activity—probably mediated by large viral dsRNAs or intramolecular base-pairing structures released from infected cells—is an essential component of the immune response elicited by virus infection in flies. The precise nature of the RNAi spread intermediate remains to be defined further.

It is remarkable that blocking the spread of the RNAi signal has such a profound effect on antiviral immunity. This suggests that the cell-autonomous RNAi response is insufficient to control a viral infection. In striking parallel to vertebrates, flies also rely on systemic immunity, albeit in this case the virus-specific signal is dsRNA-based. These observations provide an insight into the evolutionarily conserved principles of immunity in multicellular organisms, requiring both cell-autonomous responses as well as systemic mechanisms to create pre-existing immunity to protect uninfected cells.

## METHODS SUMMARY

**Fly stocks.** dsRNA uptake mutant stocks were obtained from the Bloomington *Drosophila* Stock Center. The genomic structure of these mutant allele stocks was confirmed by inverse PCR and sequencing. The *egh<sup>EP804</sup>* allele is a P-element insertion in the coding sequence of the *egh* first exon. The *ninaC<sup>3</sup>* allele is a replacement of the K1078 codon by a stop codon<sup>18</sup>. The *CG4572<sup>c05963</sup>* allele is a PiggyBac insertion in the open reading frame of *CG4572<sup>c05963</sup>*. The UAS>IR[*Ecr*] transgene producing *Ecr* dsRNA<sup>13</sup> and the P[GAL4-*ninaE.GMR*]12 GAL4 driver were recombined on chromosome 2 before genetic crosses with *egh<sup>EP804</sup>* and *CG4572<sup>c05963</sup>* mutant stocks. The GMR>IR[*white*] inverted repeat transgene has been previously described<sup>14</sup>. The Tub-eGFP transgenic line was obtained from S. Cohen<sup>19</sup>.

**dsRNA preparation and injection into adult flies.** dsRNA was generated by *in vitro* transcription using T7 RNA polymerase. Five-day-old female flies were injected in the thorax using a nanoinjector (Nanoject II, Drummond Scientific). Two days later flies were infected in the opposite side of the thorax with the appropriate virus.

**Viruses.** Recombinant Sindbis virus expressing GFP during viral replication was generated by cloning enhanced GFP into the XbaI site of the double subgenomic Sindbis vector pTE3'2J (provided by C. Rice)<sup>20</sup>. *In vitro* transcribed RNA was transfected into BHK-21 cells. Virus titre was determined by plaque assay on BHK cells. DCV stock preparation and viral titre calculation have been previously described<sup>6</sup>.

at a distal site of infection. Flies expressing eGFP (Tub-eGFP) inoculated with Sindbis-GFP (**b**, **c**) or Sindbis-luciferase virus (**d**) by intrathoracic inoculation. **b**, Viral replication monitored by RT-PCR using primers that amplify NSP1/2 virus genes. **c**, Expression of endogenous GFP was monitored by western blot with an anti-GFP antibody. **d**, Same as **c** except that flies were infected with Sindbis-luciferase virus.

**Microbial infection.** Log phase of growth cultures of bacteria *Erwinia carotovora* and *Micrococcus luteus* were re-suspended in culture medium, and sharpened needles dipped into these suspensions. Flies were harvested at 6 and 36 h after septic injury. RNA extraction and northern blots were performed following standard procedures.

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Author Contributions** M.-C.S., M.T. and R.P.v.R. performed dsRNA inoculations and virus infections, normal and reverse northern blotting, western blotting, survival curves, obtained fluorescent images, and prepared and analysed mutant flies. B.G. and V.G. examined systemic spread of dsRNA. The genetic and phenotypic analyses of transgenic flies expressing RNA hairpins were designed and carried out by B.B., C.J. and C.A. M.-C.S. and R.A. designed the experiments, discussed the interpretation of the results and co-wrote the manuscript.

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## METHODS

**Cells, plasmids and viruses.** *Drosophila* S2 cells (Invitrogen) were cultured at 25 °C in Schneider's *Drosophila* medium (Gibco), supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U ml<sup>-1</sup> penicillin, and 100 mg ml<sup>-1</sup> streptomycin. Firefly (*Photinus pyralis*) and *Renilla reniformis* luciferase sequences from the plasmids pGL3 and pRL-CMV (Promega) were cloned into pMT/V5-HisB (Invitrogen), generating pMT-Luc and pMT-Ren allowing copper-inducible expression from a metallothionein promoter.

Transfections were performed using Effectene transfection reagent (Qiagen) according to the manufacturer's recommendations. Luciferase expression was assayed using the Dual-Luciferase Reporter Assay System (Promega) and analysed on a Tecan Ultra-evolution plate reader. Double-stranded RNA was generated by *in vitro* transcription from T7-promoter-flanked PCR products. DCV viral stocks were prepared on low-passage S2 cells and titred by end-point dilution. Briefly, 25,000 S2 cells per well in a 96-well plate were inoculated with tenfold dilutions of viral stocks. Cells were transferred to fresh medium at day 7 and cytopathic effect was monitored visually over 14 days. Viral titres were calculated according to the method of Reed and Muench<sup>21</sup>.

Recombinant Sindbis virus expressing GFP during viral replication was generated by cloning enhanced GFP into the XbaI site of the double subgenomic Sindbis vector pTE3'2J (provided by C. Rice)<sup>20</sup>. The resulting plasmid was linearized and *in vitro* transcribed using the mMessage machine kit (Ambion). RNA was purified and electroporated into BHK-21 cells, and supernatant was harvested and virus titre determined by plaque assay on BHK cells.

**RNAi in S2 cells.** The effect of downregulating NinaC, CG4572 and Egghead on dsRNA uptake was analysed in a silencing of luciferase expression assay. Cells were pre-treated with approximately 500-nucleotide-long dsRNA targeting *egh* (nucleotides 488–1103; 616-bp product), *ninaC* (nucleotides 161–761; 601-bp product), *cg4572* (nucleotides 61–731; 671-bp product), or *Ago2* (nucleotides 214–865; 652-bp product), or with dsRNA targeting GFP as a negative control. Three days after knockdown of these gene products, the cells were co-transfected with an RNAi dual reporter system, consisting of firefly luciferase and *Renilla* luciferase expression plasmids. Then, dsRNA directed against firefly luciferase (nucleotides 66–658; 592-bp product) was either added to the culture supernatant (soaking) or directly introduced into cells by co-transfection with the dual reporter plasmids (transfection). Twenty-four hours after dsRNA luciferase treatment, expression of luciferase was induced by adding CuSO<sub>4</sub> to the culture supernatant, and cell lysates were generated after an additional 18 h incubation.

**Microbial infection.** The bacteria *Erwinia carotovora* and *Micrococcus luteus* were pre-cultured in LB medium. Pellets taken when the cultures were in the log phase of growth were re-suspended in a small amount of culture medium, and sharpened needles dipped into these suspensions. Flies were harvested at 6 and 36 h after septic injury. Total RNA extraction and northern blots were performed following standard procedures.

**Fly infections.** Flies were reared on standard medium at 25 °C. *Ago2*<sup>414</sup> and *Dcr2*<sup>L811f5X</sup> flies have been described previously<sup>22</sup>. *w*<sup>1118</sup> flies were used as wild-type controls. Two-to-three-day-old female flies were injected with 50 nl of the appropriate virus dilution in 10 mM Tris-Cl (pH 7.5) as described previously<sup>23</sup>, using a Drummond nanoject injector. Fly mortality at day one was attributed to damage invoked by the injection procedure, and these flies were excluded from further analyses. Mortality was monitored daily for 14 days, and every three to four days the flies were transferred to fresh food. In all experiments 40–60 flies per genotypic group were injected. Unless noted otherwise, female flies were used. No significant difference in survival was observed between flies after injection of buffer (data not shown). For northern blots, RNA was isolated from 25 flies using

Trizol reagent. Viral titres in the flies were determined by end-point dilution of fly homogenate of three pools of five flies. At the indicated time points, flies were harvested and stored at -70 °C until further processing. We confirmed the absence of endogenous virus in fly stocks by titration of uninfected fly homogenate on S2 cells.

**dsRNA preparation and injection into adult flies.** dsRNA was generated by *in vitro* transcription using T7 RNA polymerase using as template PCR products corresponding to nucleotides 1211–2112 (NSP1/2) and 5485–6439 (NSP3/4) of the Sindbis virus genome, or nucleotides 5589–6030 of the DCV genome. Five-day-old female flies were CO<sub>2</sub>-anaesthetized and injected in the thorax with 50 nl of the appropriate dsRNA (5 mg ml<sup>-1</sup>) using a nanoinjector (Nanoject II, Drummond Scientific). Two days later flies were CO<sub>2</sub>-anaesthetized and injected in the opposite side of the thorax with the appropriate virus dilution in 10 mM Tris-Cl (pH 7.5). Injection of the same volume of 10 mM Tris-HCl, pH 7.5, was used as a control. Age of the flies and amount of dsRNA injected was determined according to ref. 24. Virus infection has been described previously<sup>6</sup>.

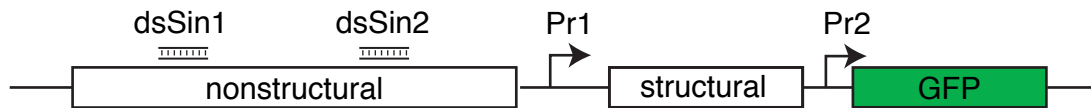
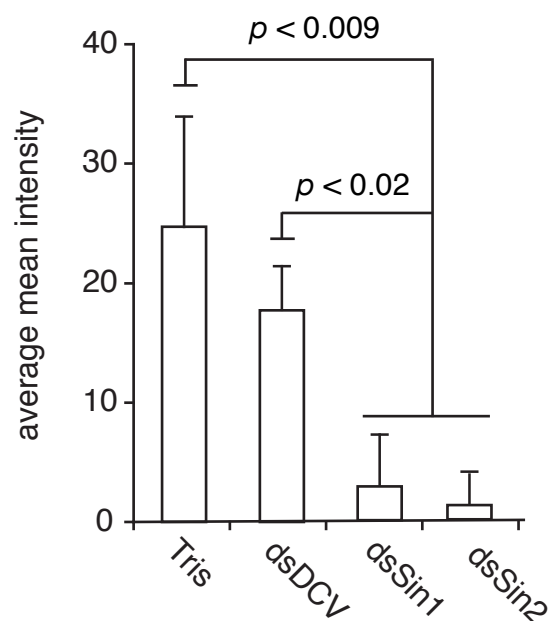
**Western blot analysis.** For protein analysis, equal amounts of protein from total fly extracts were boiled in Laemmli buffer and loaded on 10% SDS-PAGE. After transfer nitrocellulose membranes were blocked in 5% milk, 1× PBS, 0.1% Tween, and incubated overnight with rabbit polyclonal anti-GFP (Santa Cruz Biotechnology) or rabbit polyclonal anti-VP1 (custom made). For normalization a monoclonal antibody anti- $\alpha$ -tubulin (Sigma Aldrich) was used. Detection was performed using Supersignal West Pico Chemiluminescent Substrate (Pierce).

**Northern blots.** Total RNAs were extracted from whole flies using Trizol (Invitrogen). 15  $\mu$ g of total RNA was size fractionated on 1% (w/v) agarose gels containing 1.1 mM formaldehyde. After electrophoresis, the RNA was transferred overnight by capillarity to a nylon membrane (Nytran Supercharge; Schleicher and Schuell) and covalently bound to the membrane using a Stratalinker UV crosslinker. Northern blots were hybridized with DNA probes generated by a random-primed labelling reaction and [ $\alpha$ -<sup>32</sup>P]dCTP. Membranes were exposed overnight to a PhosphorImager screen at room temperature. Viral RNA was detected by northern blot using standard procedures with a random primed DNA probe corresponding to nucleotides 1947–2528 of DCV.

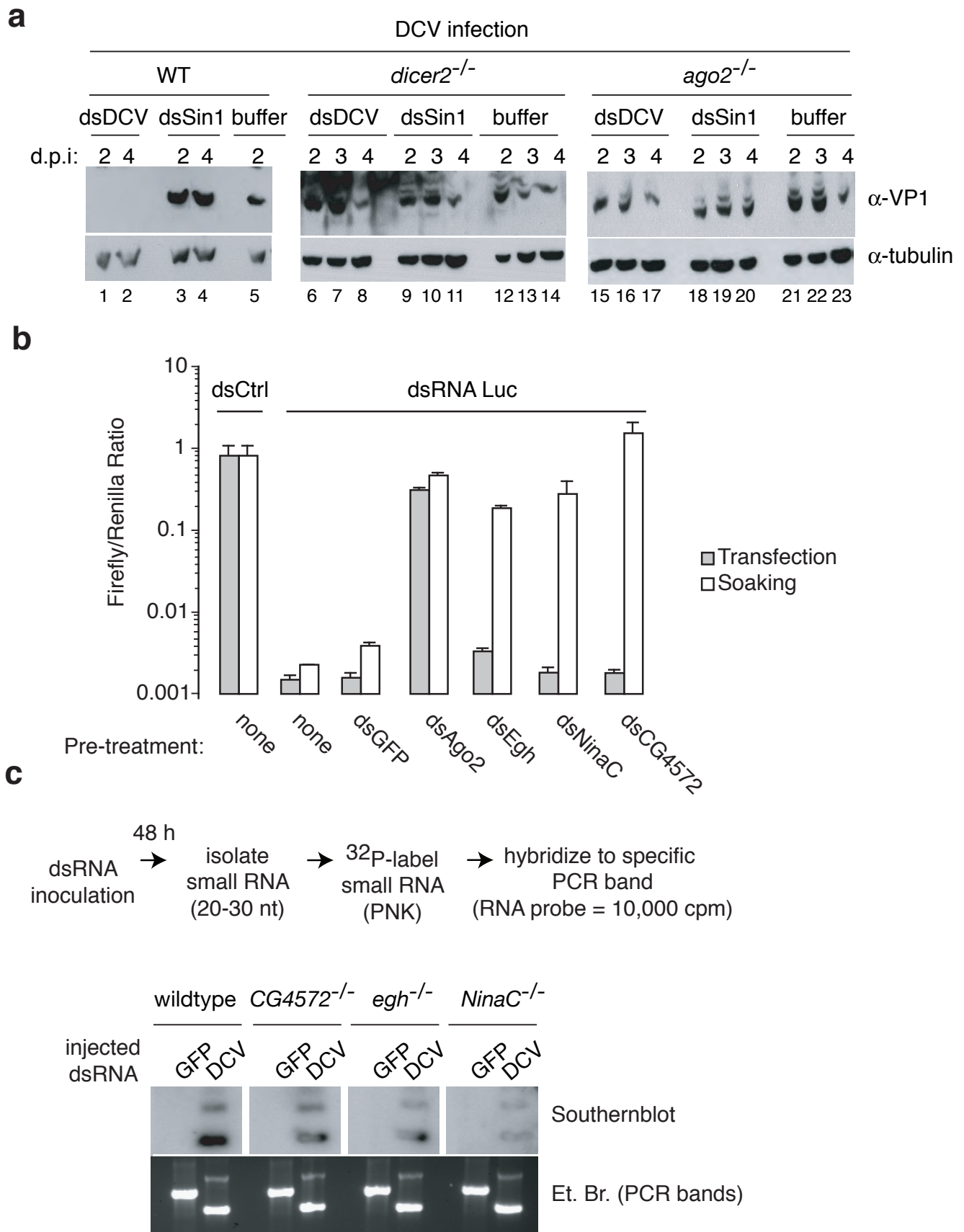
**Oligonucleotide primers.** All the primers used to produce dsRNA had a T7 promoter sequence (TAATACGACTCACTATAGGGAGA) at the 5' end. DCVpol forward, 5'-CAACGAATATGTCGCCTTGA-3'; DCVpol reverse, 5'-TTGGTTGTACGTCAAATCTGAG-3'; SINsp1 forward, 5'-TCTGCCGATCATAGCACAAAG-3'; SINsp2 reverse, 5'-CCTTCTTAACGCAACGCTTC-3'; SINsp3 forward, 5'-GAGGATCAATTTTCGACGGAGA-3'; SINsp4 reverse, 5'-GATTGAATGTCGCTGAGTCCAG-3'; *vir-1* forward, 5'-TTCGATTCCTCAGACGATGA-3'; *vir-1* reverse, 5'-GGTCAATGGGCACAAAGTTC-3'; *Rp49* forward, 5'-AAGGGTATCGACAACAGAGTGC-3'; *Rp49* reverse, 5'-ACAAATGTGTATTCCGACCACG-3'.

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## SUPPLEMENTARY INFORMATION

**a****b**

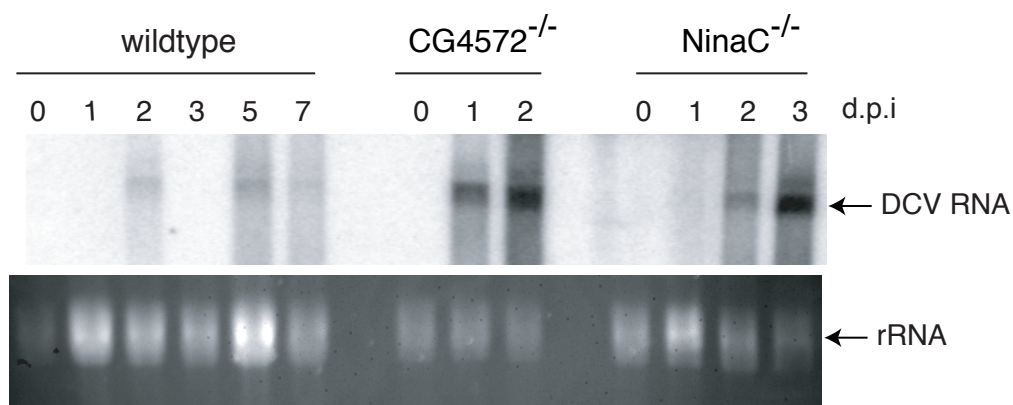
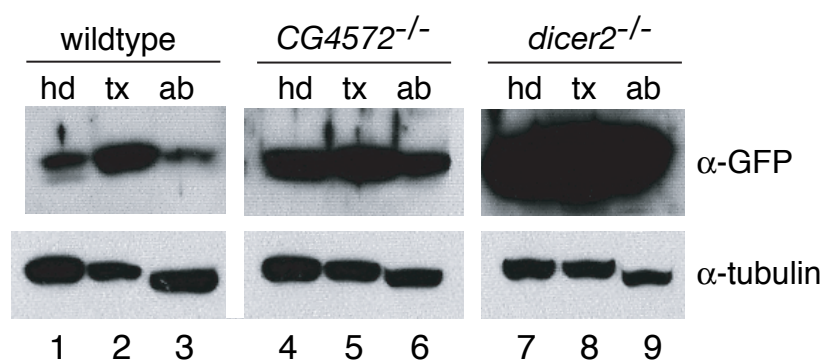
**Figure S1: Sindbis-GFP infection of *Drosophila melanogaster*.** (a) Genome organization of Sindbis-GFP recombinant virus. dsRNA used against two different regions of the nonstructural proteins (dsSin1 and dsSin2) are indicated. (b) Intensities of GFP fluorescence per group of injected flies. Flies in each group were CO<sub>2</sub>-anesthetized and imaged. Images were imported into the Volocity software (Improvision, UK) under the Measurements module. Non-injected flies were used as control background for further calculations. Data represents means and standard deviations of four to six individual flies per experiment.



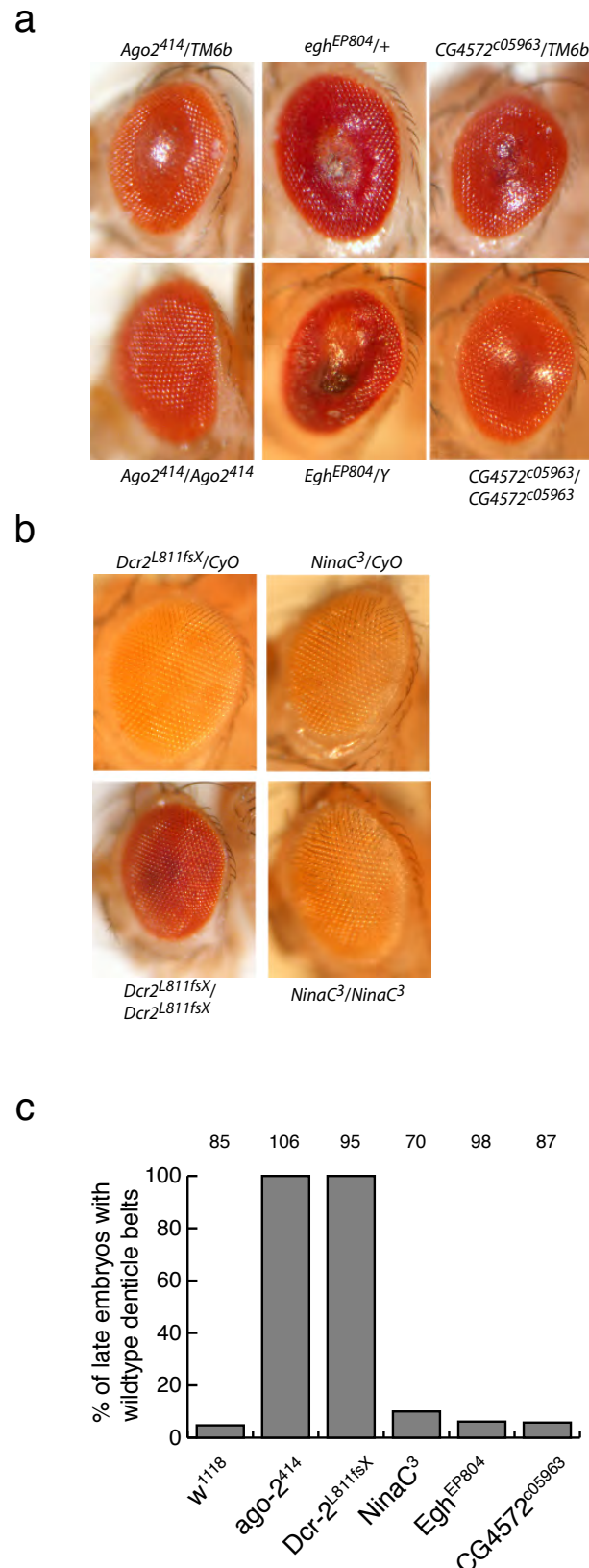
**Figure S2: dsRNA immunization provides efficient and sequence-specific antiviral protection in *D. melanogaster*** - see next page for full legend.



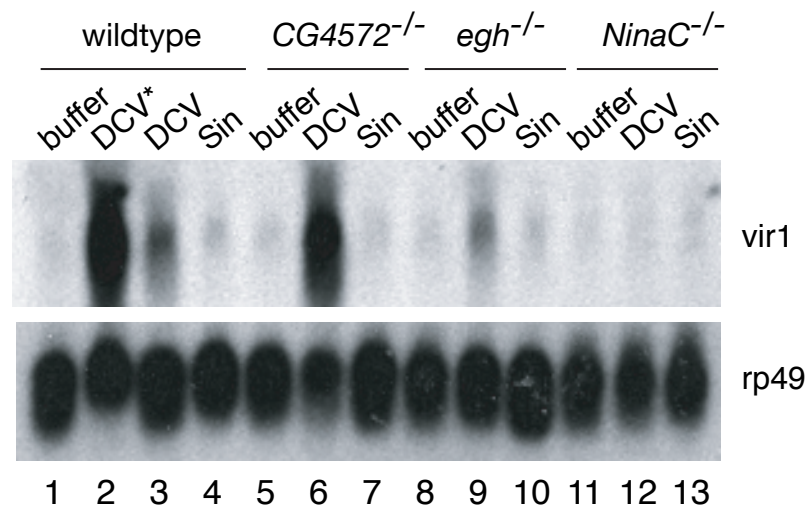
**Figure S2: dsRNA immunization provides efficient and sequence-specific antiviral protection in *D. melanogaster*.** (a) To explore the specificity of the antiviral response, we next examined the protective effect of naked dsRNA inoculation against *Drosophila* C virus, a natural pathogen of *Drosophila*. Wild type flies were intrathoracically injected with either dsRNA corresponding to DCV (dsDCV) or Sindbis virus (dsSin1); or with buffer; followed by a challenge infection with DCV. Virus replication was monitored by examining production of DCV capsid protein VP1. dsSin1, which was very effective in protecting against Sindbis virus infection, was unable to restrict DCV replication. In contrast, dsDCV effectively inhibited DCV VP1 expression (compare lanes 1 and 2 with lanes 3 and 4). This inhibitory effect was dependent on a functional RNAi machinery, because inoculation of dsDCV into *dicer-2* or *ago-2* defective flies abolished the antiviral protection (lanes 6-14 and 15-23). These results demonstrate that exogenous dsRNA can induce an effective RNAi response in *Drosophila melanogaster* that protects flies against infection by diverse viral species in a virus-specific manner. (b) Silencing of luciferase expression in *Drosophila* S2 cells depleted in dsRNA uptake genes (*egh*, *NinaC* and *CG4572*) after exposure to luciferase dsRNA by either transfection (filled bars) or by soaking dsRNA in the culture supernatant (empty bars). dsGFP: dsRNA directed against GFP, used as a negative control. dsAgo2: Ago2 dsRNA control for the core RNAi machinery depletion. Ctrl(-): untreated control. (c) Cells were pre-treated with dsRNA targeting either *egh*, *NinaC*, *cg4572*, or *ago2*, or with dsRNA targeting GFP as a negative control. Three days after knock down of these gene products, the cells were co-transfected with an RNAi dual reporter system, consisting of firefly luciferase and Renilla luciferase expression plasmids. Then, dsRNA directed against firefly luciferase was either added to the culture supernatant 'soaking' or directly introduced into cells by co-transfection with the dual reporter plasmids ('transfection'). Downregulation of the catalytic component of RISC, Ago2, inhibited RNAi silencing under each condition. Importantly, downregulation of *egh*, *NinaC* and *CG4572* impaired luciferase silencing only when dsRNAs targeting luciferase were added to the medium, but not after direct transfection into cells. This indicates that the RNAi core machinery remained functional but dsRNA uptake was impaired upon knock down of *egh*, *NinaC*, or *CG4572*. (c) Production of siRNAs derived from inoculated dsRNA is reduced in dsRNA uptake mutants. Wild type and mutant flies (*egh*<sup>-/-</sup>, *NinaC*<sup>-/-</sup>, *CG4572*<sup>-/-</sup>) were injected with dsRNA against DCV. To quantify siRNA production, small RNAs were extracted from inoculated flies, purified and radiolabelled for use as Southern blot probes. PCR product (PCR bands) corresponding to either the injected dsRNA or GFP was resolved on an agarose gel (bottom panel) and transferred to a nylon membrane. The membrane was then probed with the labelled small RNAs from inoculated flies.

**a****b**

**Figure S3: DCV and Sindbis virus replicate at higher levels in dsRNA uptake mutant flies.**  
**(a)** Northern blot analysis of DCV viral RNA. As a loading control, the ethidium bromide-stained agarose gel of total RNA preparation. Numbers at the top of the panels indicate days post-infection. **(b)** Sindbis GFP virus replicates at higher levels in mutant flies. To monitor replication of Sindbis, wild type, CG4572<sup>-/-</sup> and dicer2<sup>-/-</sup> mutant flies were infected with Sindbis-GFP and analyzed by western blotting with an anti-GFP antibody.

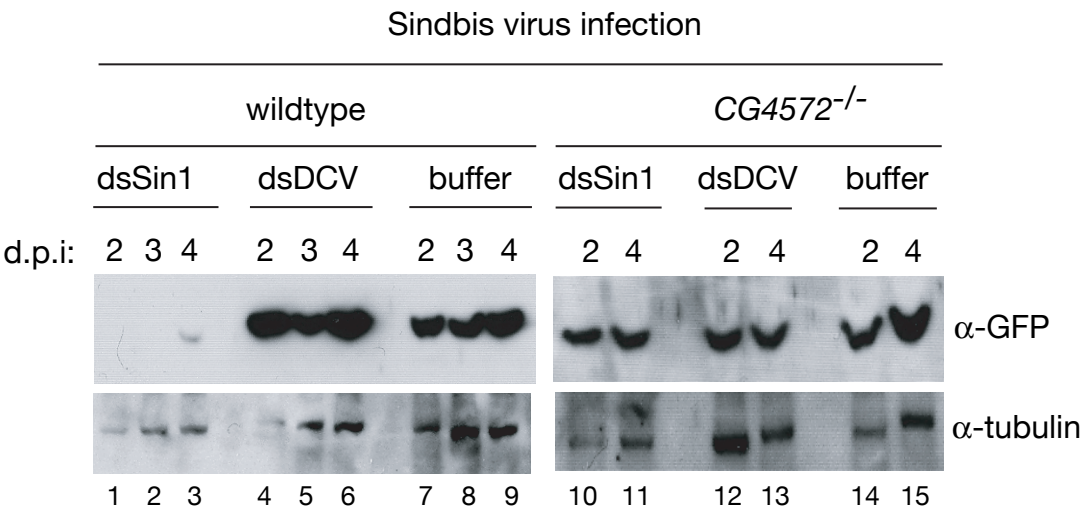


**Figure S4. Mutations in *egh*, *CG4572* and *ninaC* do not disrupt RNAi triggered by endogenous inverted repeat expression.** (a) Expression of an UAS-IR[EcR] transgene under the control of a GMR-GAL4 driver triggers RNAi in eyes of heterozygous *Ago2<sup>414</sup>*, *egh<sup>EP804</sup>* and *CG4572<sup>c05963</sup>* (upper panels), resulting in a loss of the corneal lens. RNAi is suppressed in homozygous *Ago2<sup>414</sup>* mutant control as shown by the recovery of normal eye structure, but not in hemizygous *egh<sup>EP804</sup>* or homozygous *CG4572<sup>c05963</sup>*. (b) Expression of a GMR-IR[w] transgene triggers RNAi in eyes of heterozygous *Dcr2<sup>L811fsX</sup>*, and *NinaC<sup>3</sup>* (upper panels), resulting in reduced pigment levels and orange eye color. RNAi is suppressed in homozygous *Dcr2<sup>L811fsX</sup>*, but not in homozygous *NinaC<sup>3</sup>* animals. (c) Mutations of genes involved in dsRNA uptake do not affect RNAi induced by dsRNA injection in embryos. Early embryos homozygous for the indicated alleles were injected with *ftz* dsRNA and scored for a *ftz* phenotype (six or fewer ventral denticle belts) 24h later. Numbers on top correspond to the total number of embryos injected.



**Figure S5: The JAK/Stat signalling pathway is induced by DCV infection in dsRNA uptake mutants.** RNA blot of *vir-1* (virus-induced RNA 1) in wild type and dsRNA uptake mutant flies injected with 500 TCID<sub>50</sub> of DCV, 5000 PFU of Sindbis-GFP virus or with buffer. Flies were harvested at 48 h after injection. Total RNA extraction and Northern blots for *vir1* and *rp49* were performed following standard procedures. Blots were done at least twice with the same results. DCV\*: additional control using 50000 TCID<sub>50</sub>. The antiviral defense through the activation of the Jak-STAT signaling pathway was not observed in *NinaC* mutant. It is not unexpected that a protein that mostly disrupts vesicle trafficking could also disrupt a signal transduction pathway.





**Figure S6: Systemic spread of dsRNA is essential for effective antiviral immunity.** Sindbis:GFP virus infection in wild type, and *CG4572<sup>c05963</sup>* (*CG4572<sup>-/-</sup>*) mutant flies inoculated with the indicated dsRNA. Sindbis replication was monitored by westernblotting using an antibody directed against GFP.